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COMMUNICATION

A Facile Reporter System for the Experimental Identification of Twin-Arginine Translocation (Tat) Signal Peptides from All Kingdoms of Life

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We have developed a reporter protein system for the experimental verification of twin-arginine signal peptides. This reporter system is based on the *Streptomyces coelicolor* agarase protein, which is secreted into the growth medium by the twin-arginine translocation (Tat) pathway and whose extracellular activity can be assayed colorimetrically in a semiquantitative manner. Replacement of the native agarase signal peptide with previously characterized twin-arginine signal peptides from other Gram-positive and Gram-negative bacteria resulted in efficient Tat-dependent export of agarase. Candidate twin-arginine signal peptides from archaeal proteins as well as plant thylakoid-targeting sequences were also demonstrated to mediate agarase translocation. A naturally occurring variant signal peptide with an arginine–glutamine motif instead of the consensus di-arginine was additionally recognized as a Tat-targeting sequence by *Streptomyces*. Application of the agarase assay to previously uncharacterized candidate Tat signal peptides from *Bacillus subtilis* identified two further probable Tat substrates in this organism. This is the first versatile reporter system for Tat signal peptide identification.

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Keywords: protein export; Tat system; twin-arginine signal peptide; reporter system; *Streptomyces*

The twin-arginine translocation (Tat) pathway is a general protein export pathway found in the cytoplasmic membranes of most bacteria and in the

thylakoid membranes of plant chloroplasts. Unlike the well-characterized Sec pathway, protein substrates of the Tat pathway are usually transported across the membrane in a folded state. In Gram-negative bacteria such as *Escherichia coli* and some Gram-positive bacteria of the actinobacterial class, for example, *Streptomyces coelicolor*, transport on the Tat pathway is achieved by three proteins, TatA, TatB, and TatC.^{1–4} Most Gram-positive bacteria, however, exemplified by *Bacillus subtilis*, and archaeobacteria

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Abbreviation used: Tat, twin-arginine translocation.

have Tat systems that only have TatA and TatC components.⁵⁻⁷ The organization of the thylakoid Tat pathway more closely resembles that of the Gram-negative bacteria, with three essential components designated Tha4, Hcf106, and cpTatC.^{8,9}

Proteins are targeted to the Tat pathway by tripartite N-terminal signal peptides, the amino-terminal portion (n region) of which contains a conserved twin-arginine motif. For eubacterial Tat signal peptides, this motif is defined as (S/T)-R-R-x-F-L-K,

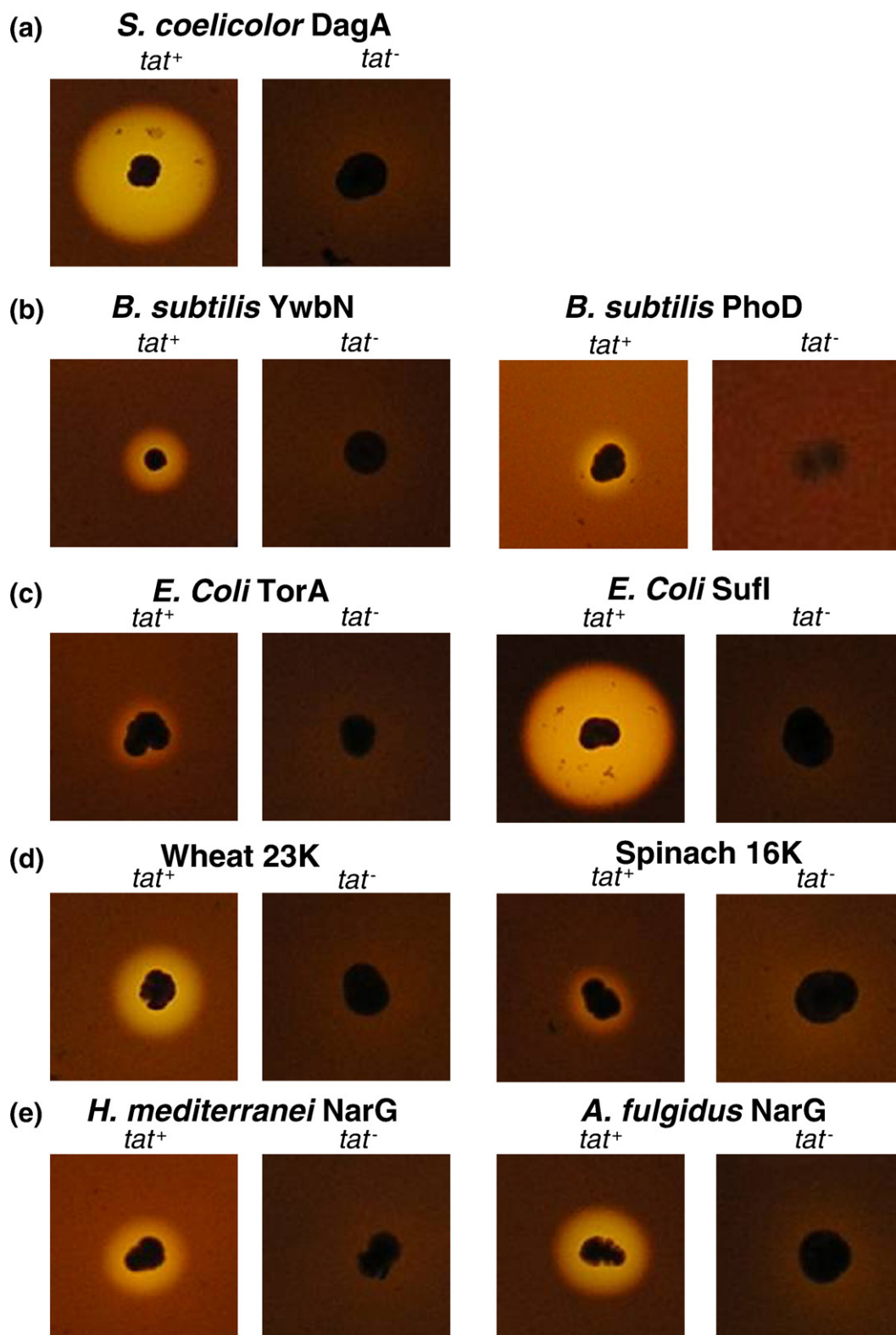


Fig. 1 (legend on next page)

where the twin arginines are usually, although not always, invariant; the other motif residues occur with a frequency exceeding 50%, and x is any polar amino acid.^{10,11} For signal peptides that direct proteins to the thylakoid Tat system, the targeting motif has been defined as R-R-x-Φ-Φ, where Φ represents a hydrophobic amino acid.^{12,13} The Tat pathways in bacteria and chloroplasts are functionally and mechanistically related (reviewed, e.g., in Refs. 14,15), and in support of this, it has been shown that bacterial Tat-targeting signals can function to direct the transport of proteins by the thylakoid Tat pathway.^{16–18} However, to date, there are no reports that eukaryotic Tat signals are functional in bacterial systems.

At least three different prediction programs that have been designed to recognize the salient features of Tat targeting sequences have been developed.^{19–21} A comparison of the two generally available programs, TATFIND and TatP, suggests that an overlapping set of candidate twin-arginine signal peptides is recognized by both programs but that, in addition, a number of potential Tat signal peptides are only identified by one method or the other. Moreover, although the consecutive arginine residues are very highly conserved, there are a few, naturally occurring, Tat-targeting signals where one of the consensus arginine residues has been replaced by lysine^{22,23} or asparagine.²⁴ These variant Tat signal peptides appear to be very rare and are much more difficult to identify by bioinformatics means alone.

Despite the availability of Tat prediction programs, there is currently no universal reporter system that can be used to verify Tat-dependent protein export by candidate signal peptides. Such a reporter would be a particularly important tool in the context of functional genomic studies of organisms that cannot be genetically manipulated or for analysis of metagenomes from uncultured bacteria. A number of Tat-dependent reporter systems have been developed, for example, based on export of green fluorescent protein,²⁵ maltose binding protein,²⁶ or chloramphenicol acetyltransferase.²⁷ However, these reporters are designed mainly for use in *E. coli*, with the *E. coli* Tat machinery often not recognizing 'foreign' Tat signal peptides being a major drawback,^{5,28} possibly because it only has a relatively small repertoire of Tat substrates. Recently, it was predicted bioinformatically^{19,20} and subsequently verified experimentally²⁹ that *S. coelicolor* has an exceptionally large number of Tat-dependent proteins. Moreover, heterologous expression of *S. coelicolor* tat components in

E. coli demonstrated that the *S. coelicolor* Tat system is permissive such that it can form functional heterologous complexes with *E. coli* components and recognize native *E. coli* Tat substrates.³⁰ Therefore, *Streptomyces* may represent a more promising host for the development of a Tat-dependent protein reporter system.

Agarase as a Tat-dependent reporter protein

We have demonstrated previously that agarase can be used as a Tat-specific reporter system in *Streptomyces* for the identification of host signal peptides.²⁹ Agarase is a secreted enzyme that degrades agar into smaller oligosaccharides,³¹ which can be readily visualized by staining agar plates with iodine, which leads to a zone of clearing around the colony (as shown in Fig. 1a). *S. coelicolor* agarase is a Tat-dependent, Sec-incompatible protein, and its export can be mediated by any of 27 *S. coelicolor* twin-arginine signal peptides to which it has been genetically fused.²⁹ Given the large numbers of Tat substrates in *Streptomyces* and the flexibility of the *Streptomyces* Tat system for heterologous interactions, we reasoned that agarase might form the basis of a reporter system for the general cross-species identification of Tat signal peptides. To test this, we initially constructed fusions of mature agarase to two Tat-targeting signal peptides from the Gram-positive organism *B. subtilis*. As discussed above, *B. subtilis* differs from *S. coelicolor* because it lacks the TatB protein. Moreover, it has two distinct Tat systems, one encoded by *tatA_d/C_d* that specifically transports the substrate PhoD and a second system designated *TatA_y/C_y* that has YwbN as a substrate.^{5,6} These constructs were transformed into a wild-type and *tatC* variant of *Streptomyces lividans*.³³ *S. lividans* was chosen as a host because unlike *S. coelicolor*, to which it is extremely closely related, it does not encode native agarase; moreover, the *S. coelicolor* agarase promoter is expressed constitutively in this strain background.³⁴

As shown in Fig. 1b, both the *B. subtilis* PhoD (two rightmost panels) and YwbN (two leftmost panels) signal peptides were able to mediate Tat-dependent export of agarase in *Streptomyces*. Quantification of the level of extracellular agarase activity, as shown in Fig. 2a, demonstrates that the YwbN signal peptide mediated the export of more enzyme than the PhoD signal peptide. Whether this reflects a difference in the level of translation of the two signal peptide coding regions or a difference in export

Fig. 1. Signal peptides from the three kingdoms of life mediate Tat-dependent secretion of *S. coelicolor* agarase. Extracellular agarase activity mediated by signal peptides from (a) *S. coelicolor* agarase (DagA), (b) *B. subtilis* YwbN (two leftmost panels) and PhoD (two rightmost panels), (c) *E. coli* TorA (two leftmost panels) and SufI (two rightmost panels), (d) wheat 23K (two leftmost panels) and spinach 16K (two rightmost panels), and (e) NarG from *H. mediterranei* (two leftmost panels) and *A. fulgidus* (two rightmost panels). Strains were cultured on MM medium [per liter: 10 g Agar, 1 g (NH₄)₂SO₄, 0.5 g K₂HPO₄, and 0.2 g FeSO₄·7H₂O] containing 0.5% glucose. The inoculated plates were grown for 5 days at 30 °C after which they were stained with Lugol's solution (Sigma). For each panel, the signal peptide of the indicated protein, fused in frame with the mature sequence of agarase as described in the legend to Table 1, was expressed in the *S. lividans* *tat*⁺ strain (10–164, designated *tat*⁺ and shown on the left of each panel set) or in the 10–164 isogenic *tatC* strain³² (designated *tat*[−] and shown on the right of each panel set).

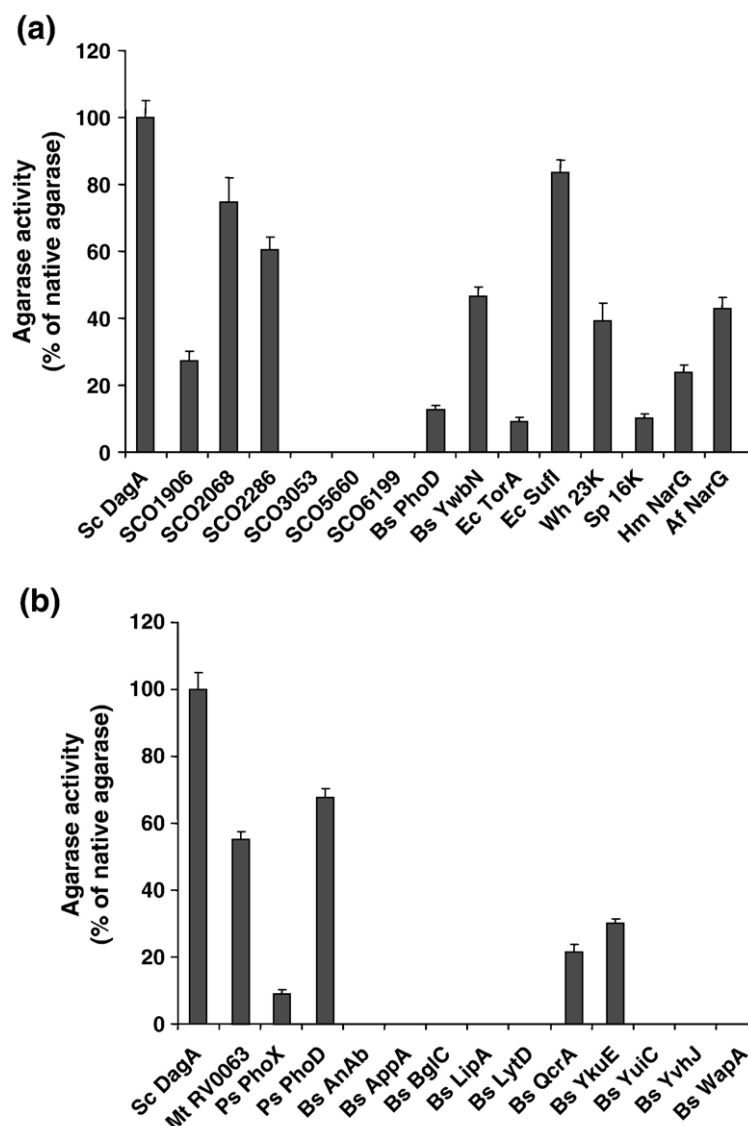


Fig. 2. Semiquantitative analysis of extracellular agarase activity mediated by twin-arginine signal peptides. Zones of clearing around colonies after Lugol staining were measured, and relative agarase activity was estimated as described in Widdick *et al.*²⁹ The error bars represent the standard error of the mean, $n=6-9$. (a) Relative agarase activity from fusion proteins harboring signal peptides from native agarase (Sc DagA), *B. subtilis* PhoD (Bs PhoD) and YwbN (Bs YwbN), *E. coli* TorA (Ec TorA) and SufI (Ec SufI), wheat 23K (Wh 23K), spinach 16K (Sp 16K), *H. mediterranei* NarG (Hm NarG), and *A. fulgidus* NarG (Af NarG). Fusions to the signal peptides of SCO1906 (*S. coelicolor* PhoX homologue) and of SCO2068 and SCO2286 (both *S. coelicolor* PhoD homologues) were included as positive controls, and fusions for the signal peptides of SCO3053, SCO5660, and SCO6199 were included as negative controls.²⁹ (b) Relative agarase activity from fusion proteins harboring signal peptides from native agarase (Sc DagA), *M. tuberculosis* RV0063 (Mt RV0063), *P. syringae* PhoX (Ps PhoX) and PhoD (Ps PhoD), and *B. subtilis* AbnA (Bs AbnA), AppA (Bs AppA), BglC (Bs BglC), LipA (Bs LipA), LytD (Bs LytD), QcrA (Bs QcrA), YkuE (Bs YkuE), YuiC (Bs YuiC), YvhJ (Bs YvhJ), and WapA (Bs WapA).

efficiency remains to be shown. Nonetheless, we conclude that despite differences in the composition of the Tat machinery and codon usage between the two organisms, *Bacillus* twin-arginine signal peptides can functionally engage with the *Streptomyces* Tat machinery.

Tat-dependent export of agarase can be mediated by signal sequences from Gram-negative bacteria, eukaryotes, and archaea

We next tested whether signal peptides from the model Gram-negative bacterium *E. coli* could mediate export of agarase. The two signal peptides we chose to test were those of TorA and SufI. TorA is the trimethylamine-*N*-oxide reductase enzyme, which allows *E. coli* to grow using trimethylamine-*N*-oxide as a sole energy source. This is the most heavily exploited Tat signal peptide (e.g., Refs. 25, 26), and heterologous expression of the *S. coelicolor* *tat* genes in *E. coli* *tat*[−] strains has indicated that this

signal peptide can be recognized by the *Streptomyces* Tat machinery.³⁰ As shown in Fig. 1c, fusion of the TorA signal peptide to agarase did indeed mediate its secretion by the Tat pathway. Analysis of the SufI signal peptide, which is another well-characterized *E. coli* Tat-targeting sequence, showed that it also directed the transport of agarase (Fig. 1c). Furthermore, as shown in Fig. 2a, quantification of agarase activity indicated that SufI was a particularly effective signal for the export of this reporter protein.

Since any effective Tat reporter system would be required to recognize Tat signal peptides across biological kingdoms, we also tested the ability of known Tat-targeting sequences from plants as well as predicted Tat signal sequences from archaea to direct export of agarase. The spinach 16K and wheat 23K proteins of the oxygen-evolving complex of plant photosystem II are model substrates of the thylakoid Tat system.^{35,36} Figures 1d and 2a demonstrate clearly that the twin-arginine signal sequences of both of these proteins were capable of

mediating transport of agarase by the *Streptomyces* Tat pathway. This important observation represents the first time that eukaryotic twin-arginine signal peptides have been shown to function in a prokaryotic system.

The archaeon *Haloferax mediterranei* has a molybdenum cofactor-containing nitrate reductase of the Nar family that has recently been strongly inferred by substrate accessibility experiments to be located at the external face of the cytoplasmic membrane.³⁷ As shown in Table 1, this protein is synthesized with a classical twin-arginine signal sequence, although it lacks an obvious signal peptidase I cleavage site and might therefore serve as a noncleaved signal anchor. When this signal sequence was fused to mature agarase, it was clearly able to mediate Tat-dependent export of the protein (Fig. 1e, two leftmost panels; Fig. 2a). *Archaeoglobus fulgidus* has a similar Nar enzyme that is synthesized with a twin-arginine signal sequence, and we also tested the ability of that sequence to export agarase. Again, as shown in Figs. 1e (two rightmost panels) and 2a, this signal is also competent to direct Tat-dependent export of agarase. We conclude that archaeal twin-arginine signal sequences are also recognized by the *Streptomyces* Tat machinery.

A naturally occurring variant Tat signal peptide can mediate export of agarase

Although the vast majority of known Tat-targeting sequences contain the signature pair of consecutive arginines, there are a few naturally occurring Tat signal sequences that lack one of the paired arginine residues. A likely candidate for a variant Tat signal is *Mycobacterium tuberculosis* RV0063, a probable flavin adenine dinucleotide-containing oxidoreductase, which has an arginine–glutamine pairing in place of the twin arginines. Homologues of RV0063 in other mycobacterial species have a similar Arg–Gln motif in their signal peptides, whereas more distantly related organisms such as *Streptomyces avermitilis* and *Frankia* sp. encode homologues with a standard twin-arginine pair. In order to test whether this variant signal sequence was recognized by the *Streptomyces* Tat system, we genetically fused the coding sequence of the signal peptide to the DNA region for mature agarase. As shown in Figs. 3a and 2b, the variant signal peptide of RV0063 directed significant export of active agarase. It should be noted that although DeLisa *et al.*²⁵ have previously shown by site-directed mutagenesis that an Arg–Gln pairing in the signal peptide of TorA was still functional for export, this

Table 1. Signal sequences tested in this study

Signal sequence source	Amino acid sequence of signal
<i>S. coelicolor</i> agarase	MVNR <u>RR</u> DLIKWSAVALGAGAGLAGPAPAAHAGS
<i>B. subtilis</i> YwbN	MSDEQKKPEQ <u>I</u> <u>RR</u> DILKWGAMAGAAVAIGASGLGLAPLVQTAGS
<i>B. subtilis</i> PhoD	MAYDSRFDEWVQKLKEESFQNNFT <u>DR</u> RKFIQAGAGKISLGLTIAQSVGAGS
<i>E. coli</i> TorA	MNNNDLFQAS <u>RRR</u> FLAQLGGLTVAGMLGPSLLTPRRATAAQAGS
<i>E. coli</i> SufI	MSLS <u>RR</u> RQFIQASGIALCAGAVPLKASAAGGS
Wheat 23K protein ^a	(M)AQKNDEAA <u>SD</u> AAVVT <u>SR</u> RAALSLLAGAAIAVKVSPAAAGS
Spinach 16K protein ^a	(M)AQQVSAAEAETS <u>RR</u> RAMLGFAAGLASGSFVKAVLAGS
<i>H. mediterranei</i> NarG	MDADSGV <u>SR</u> RTF <u>LE</u> GIGVASLLIGTSAASDDSLFQGS
<i>A. fulgidus</i> NarG	MKV <u>SR</u> RD <u>FI</u> KLSAATAFASGLGLGYFQKSRGVGS
<i>M. tuberculosis</i> RV0063	MAREIS <u>RQ</u> TFLRGAAGALAAGAVFGSVRATADPAGS
<i>P. syringae</i> PhoX	MKLLEENQTTDLENMVGASRA <u>GLT</u> <u>RR</u> GFISAGALCGAAMFLGGNLSRTVLANSVGS
<i>P. syringae</i> PhoD	MTDFNP <u>DR</u> RRITGVAGATLLSILSPFARSAGGS
<i>B. subtilis</i> AbnA	MKKKKTW <u>KR</u> FLHFSSAALAAGLIFTSAAPAEAAFGS
<i>B. subtilis</i> AppA	M <u>KRR</u> KTALMMLSVLMVLAIFLSACSGSKSSNSSAKKGS
<i>B. subtilis</i> BglC	<u>MK</u> RSISIFITCLLITLTMGGMIASPAAGGS
<i>B. subtilis</i> LipA	MKFV <u>KRR</u> IILVTLMLSVTSLFALQPSAKAAEGS
<i>B. subtilis</i> LyrD	M <u>KR</u> LIA <u>P</u> MLLSAASLAFFAMSGSAQAAAGS
<i>B. subtilis</i> QcrA	MGGKHDIS <u>RR</u> QFLNYTLTGVGGFMAASMLMPMVRFALDGS
<i>B. subtilis</i> YkuE	MKKMS <u>RR</u> QFLKGMFGALAAAGALTAGGGYGYARYGS
<i>B. subtilis</i> YuiC	MMLN <u>M</u> <u>IR</u> RLMTCLFLAFGTTFLSVSGIEAKDGS
<i>B. subtilis</i> YvhJ	MAERVVRVRK <u>KKK</u> SK <u>RR</u> KIL <u>KR</u> IMLLFALALLVVVGLGGYKLYKTINAADGS
<i>B. subtilis</i> WapA	M <u>KRR</u> K <u>RR</u> N <u>FR</u> K <u>FI</u> AFLVLA <u>MIS</u> LPADVLAKSGS

The consecutive arginines of the twin-arginine motif (or naturally occurring variants thereof) are in boldface and underlined. Signal peptide–agarase fusions were constructed in vector pTDW46.²⁹ Signal peptides were amplified using the primers listed in Supplementary Table 1, digested with NdeI and BamHI, and cloned into pTDW46 that had been similarly digested. The six base pairs of the BamHI site add the amino acids Gly–Ser to the end of each signal peptide coding sequence; therefore, in the table, each sequence ends with GS. The native agarase signal peptide was cloned in an identical manner²⁹ and also has the GS motif at its C-terminal end. For signal peptides encoded by *B. subtilis*, *P. syringae*, or *E. coli*, the coding region was amplified by PCR using the appropriate chromosomal DNA as template. The remaining signal peptides were constructed by annealing long oligonucleotides having complementary 3' ends and extending them with *taq* polymerase to make long double-stranded oligonucleotides encoding the desired signal peptides. All plasmids were verified by DNA sequencing and then subsequently transferred by mating from *E. coli* into the *S. lividans* *tat*⁺ and *tat*[−] strains where they integrate in single copy at the phage ϕ C31 site.

^a Nuclear-encoded proteins targeted to the thylakoid Tat system are synthesized with an N-terminal chloroplast-targeting sequence that effects their translocation into the chloroplast. This is subsequently cleaved off by a stromally located peptidase to reveal an N-terminal Tat-targeting sequence. This sequence does not normally start with a methionine; however, an N-terminal methionine was included in this case to enable translational initiation.

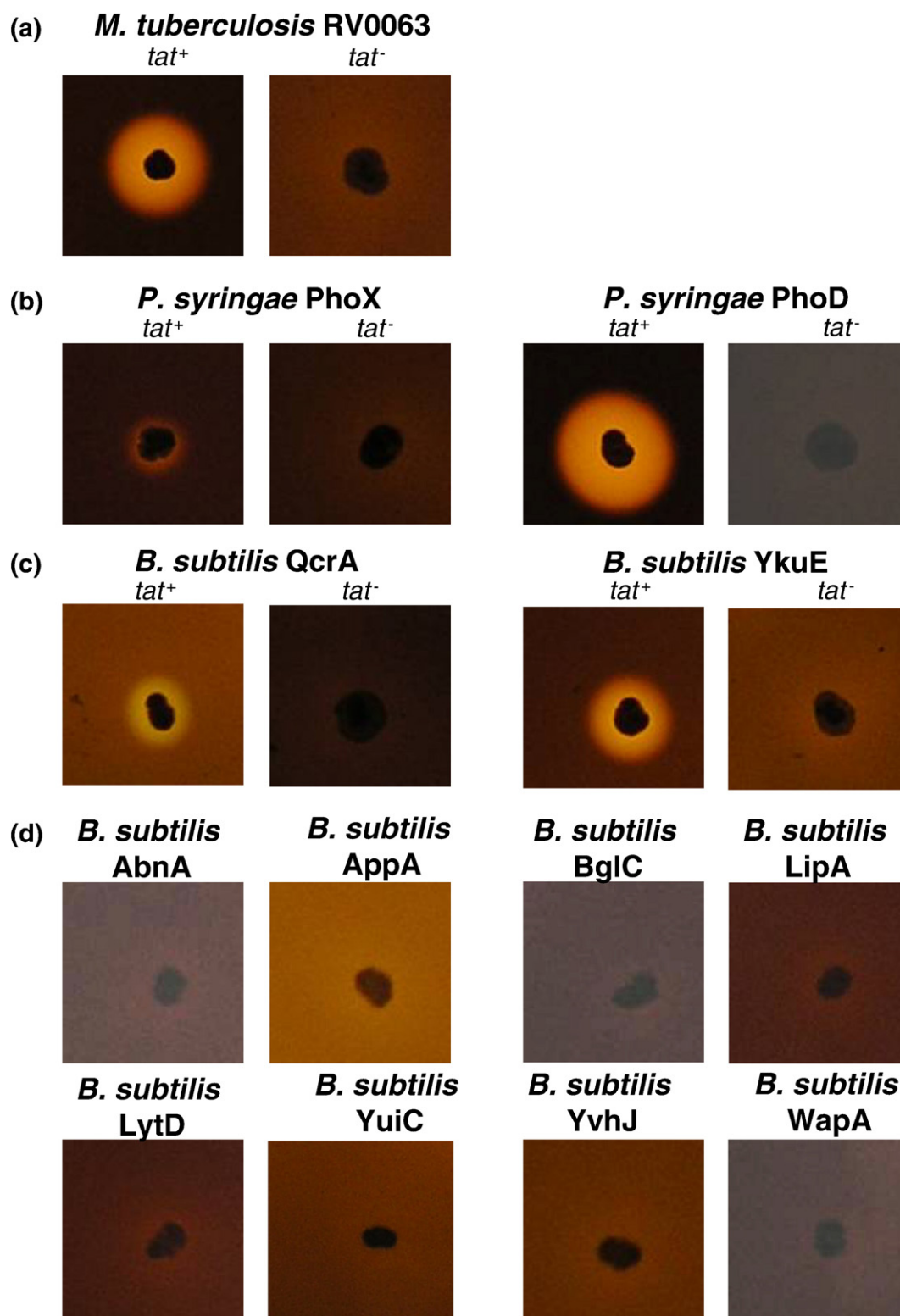


Fig. 3. Experimental verification of uncharacterized and variant Tat-targeting sequences using the agarase reporter system. (a–c) The signal peptide of the indicated protein, fused in frame with the mature sequence of agarase as described in the legend to Table 1, was expressed in *S. lividans* *tat*⁺ strain (10–164, designated *tat*⁺ and shown on the left of each panel set) or in the 10–164 isogenic *tatC* strain³³ (designated *tat*⁻ and shown on the right of each panel set). The signal peptides tested are (a) *M. tuberculosis* RV0063, (b) *P. syringae* PhoX (two leftmost panels) and PhoD (two rightmost panels), and (c) *B. subtilis* QcrA (two leftmost panels) and YkuE (two rightmost panels). (d) The signal peptides of *B. subtilis* AbnA, AppA, BglC, LipA, LytD, YuiC, YvhJ, and WapA were fused in frame to agarase, but the results are shown for the *S. lividans* *tat*⁺ strain only.

is the first example of a natural occurrence of this sequence in a Tat substrate. These observations confirm that the agarase reporter system is a robust method for the verification of Tat-targeting sequences.

Application of the agarase reporter system for the experimental verification of Tat signal peptides from *Pseudomonas syringae* and *B. subtilis*

Since we have demonstrated that the agarase assay has general utility as a reporter system for Tat signal peptides, we set about using it to look for Tat-targeting sequences from the genomes of two bacteria. The Tat system of the Gram-negative plant pathogen *P. syringae* pv. tomato strain DC3000 has previously been analyzed and shown to be functional and to be required for optimal virulence.^{38,39} *P. syringae* DC3000 encodes phosphatase proteins of both the PhoD and PhoX family; *S. coelicolor* orthologues of both of these proteins and the *B. subtilis* orthologue of PhoD have been previously shown to be Tat substrates. Unusually, the *P. syringae* PhoX signal sequence has a highly extended n region prior to the twin-arginine motif (Table 1), which is not present on the signal sequence of the *S. coelicolor* orthologue. Nonetheless, this signal peptide was capable of directing export of agarase in *Streptomyces* (Fig. 3b, two leftmost panels). Conversely, while the signal peptides of PhoD proteins are generally very extended, the *P. syringae* PhoD signal is predicted to be unusually short (Table 1) and is not recognized *in silico* as a Tat signal peptide by TatP, although it does pass the criteria for positive identification by the TATFIND program. This signal peptide was also functional in the Tat-dependent export of *Streptomyces* agarase (Fig. 3b, two rightmost panels). Quantification of agarase activity, as shown in Fig. 2b, indicates a high level of export mediated in particular by the *P. syringae* PhoD signal sequence.

Remarkably, despite the fact that it has two distinct Tat systems, only two Tat substrates have been confirmed in *B. subtilis*, that is, PhoD and YwbN, and we have demonstrated above that each of these bears Tat-targeting signals that are functional in *Streptomyces*. Application of TATFIND or TatP predicts that there are very few Tat signal peptides encoded in the genome of *B. subtilis*, although using less stringent criteria, for example, allowing for a more hydrophobic h region or the presence of a lysine in place of the first arginine residue, generates more significant lists.^{40–42} We therefore selected 10 candidate Tat signal peptides, shown in Table 1, from the list generated by Jongbloed *et al.* for testing in the agarase reporter assay.⁴¹ As shown in Figs. 3c and 2b, two of the signal peptides we tested, those of QcrA and YkuE, were able to direct export of *S. coelicolor* agarase, and we therefore conclude that these are *bona fide* Tat-targeting sequences. The remaining signal peptides tested, as shown in Fig. 3d, did not mediate export of agarase, and it is therefore unlikely that any of these are Tat signals. Of

these, it has already been shown that AbnA, BglC, LipA, LytD, and WapA are Sec substrates because they are still secreted even when both Tat systems from *B. subtilis* have been inactivated.⁴¹

One of the two signal peptides that scored positive for agarase export is derived from QcrA, which is the *B. subtilis* homologue of the Rieske iron sulfur protein, a known Tat substrate in plants²³ and recently also shown to be a Tat substrate in the bacteria *Legionella pneumophila* and *Paracoccus denitrificans*.^{32,43} Interestingly, the Rieske proteins have uncleaved signal anchors and this seems to be true also for *B. subtilis* QcrA since the consensus signal peptidase cleavage motifs are absent from this protein.⁴⁴ Our findings therefore suggest that normally uncleaved twin-arginine signal peptides may also be positively identified as Tat-targeting sequences with the agarase reporter system. Whether this is a general feature of this reporter system awaits further verification. Taken together, our results indicate that there are at least four Tat substrates in *B. subtilis*.

In conclusion, we have demonstrated the utility of *S. coelicolor* agarase as a general reporter for twin-arginine signal peptides from a wide range of organisms. Our studies show that export of agarase in *Streptomyces* can be mediated by signal peptides derived not only from other Gram-positive organisms but also from Gram-negative bacteria, archaea, and even eukaryotes. This reporter system provides an important first step in establishing the Tat dependence of an exported protein, which should then be confirmed in the natural host where possible. This would ultimately be desirable since it has been shown that, in some instances, the nature of the mature domain can influence the export pathway with which a putative Tat signal engages (e.g., Ref. 45), although we note that this may be possible for only a limited number of organisms. Moreover, since the agarase assay is semiquantitative, it permits the identification of Tat-targeting sequences that function efficiently in *Streptomyces* (with the caveat that this does not necessarily indicate that the signal peptide functions efficiently in the natural host) and allows for more detailed characterization of the minimal requirements in the Tat signal sequence by random or site-directed mutagenesis. Thus, the agarase reporter system is facile, sensitive, and broad ranging and represents a major new tool in the characterization of Tat systems.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2007.11.002](https://doi.org/10.1016/j.jmb.2007.11.002)

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